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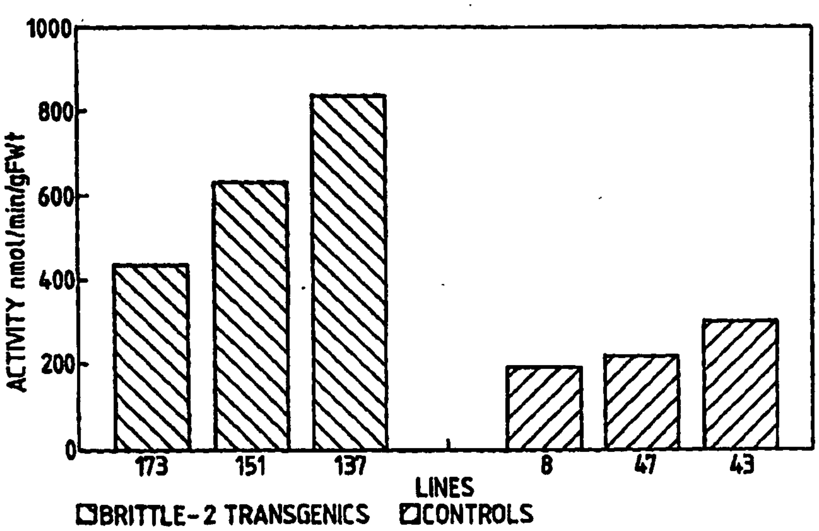

(57) Abstract

This invention is based on the surprising effect of increased enzyme activity when one of the genes of one of the subunit proteins of an enzyme catalysing starch is incorporated into a plant. The method also includes the introduction of one of the genes of one of the subunit proteins of a plurality of other enzymes catalysing starch synthesis. The gene is suitably a *brittle-2* gene. Increased activity of ADPG PPase is shown and also an increase in starch synthesis.

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Modification of Starch Content in Plants

This invention relates to the modification of starch content of plants, and in particular, to the increase of starch content in plants.

Starch is a complex polymer of glucosyl residues. It is the major form in which carbohydrate is stored in the tissues of most species of higher plants. It is accumulated in the leaves of plants during the day as a result of photosynthesis and is used to supply the needs of the plant for energy and biosynthesis during the night. Starch is also accumulated in non-photosynthetic tissues, especially those involved in reproduction such as seeds, fruits and tubers. Therefore, starch is of great importance to the productivity of the plant and its survival.

Starch is also highly significant to man. Firstly, it forms a major component of animal diets, supplying man and his domestic animals with a large portion of their carbohydrate intake. Secondly, the type of starch in a plant affects the quality of the processed plant product. Thirdly, starch is used industrially in the production of paper, textiles, plastics and adhesives, as well as providing the raw material for some bio-reactors. Starch from different species have preferred uses. On a world scale, starch producing crops are agriculturally and economically by far the most important, and these crops include wheat, maize, rice and potatoes. The quantity of

starch present in the harvested organ of a plant will affect the gross yield and the processing efficiency of the crop. In addition, the type of starch will affect the quality of a processed product and the profitability of the process.

Starch is synthesised in amyloplasts in plants from glucose-1-phosphate (Glc-1-P) as shown below.

Glucose-1-P

$\downarrow$ ADPG PPase

Adenosine diphosphoglucose (ADPG)

$\downarrow$ Starch synthase and branching enzyme

Starch [Amylose and Amylopectin]

Adenosine diphosphoglucose pyrophosphorylase [EC.2.7.7.27] (ADPG PPase) catalyses the first committed step of the pathway of starch biosynthesis in plants. A similar enzyme catalysing the same reaction is found in bacteria and cyanobacteria.

The quaternary structure of the enzyme is similar in all organisms investigated in that the functional enzyme is composed of a tetramer of subunit proteins. In bacteria the protein subunits are identical and the product of a single gene, e.g. in *E. coli* the GlgC gene. In plants, however, the enzyme is composed of two each of two different protein subunits. While these different protein subunits display sequence similarities, they are the product of two distinct genes.

There are many mutants of plants that have a lower starch content in particular tissues compared to that of wild-type plants. These mutant plants are deficient in

the expression of one of the genes coding for the subunits of ADPG PPase. Two particular mutations seen in maize endosperm are the mutants *shrunken-2* and *brittle-2*. It is argued that the wild-type genes for these code for the two subunit proteins of the enzyme. Both mutations cause decreased enzyme activity of ADPG PPase in the endosperm. It is argued from this information that both subunits of the enzyme are required for full activity and that lack of a particular type of subunit cannot be compensated for by the other subunit.

This invention is based on the fact that one only of the genes for one of the subunit proteins of an enzyme catalysing starch production is required to increase enzyme activity.

It is an object of the present invention to provide a method for increasing the activity of an enzyme catalysing starch synthesis.

It is a further object of the present invention to provide a plant having an increased starch content when compared with a control plant not treated in accordance with the inventive method.

It is also an object of the invention to increase the rate of starch synthesis under conditions which do not lead to a compensating increase in the rate of starch breakdown.

The present invention provides a method of increasing the enzyme activity in a plant comprising introducing into a plant one of the genes of one of the subunit proteins of an enzyme catalysing starch synthesis, thereby causing

expression of the subunit gene in the plant to produce the subunit protein, and an increase in the enzyme activity in the plant cells.

The present invention further provides a plant into which has been introduced one of the genes of one of the subunit proteins of an enzyme catalysing starch synthesis, which plant expresses the gene to produce a subunit protein, and increases the enzyme activity in the plant cells.

The method may also include introducing one of the genes of one of the subunit proteins of a plurality of other enzymes catalysing starch synthesis.

The present invention also provides a plasmid incorporating one of the genes of one of the subunit proteins of an enzyme catalysing starch synthesis in plants.

A plasmid or plant according to the invention may also contain one of the genes of one of the subunit proteins of one or more other enzymes catalysing starch synthesis in the plants.

The present invention also provides a plant cell harbouring a plasmid described above.

Preferably the gene is the *brittle-2* gene or a homologue thereof. Advantageously the gene is the wheat *brittle-2* gene.

Preferably the plant is grown commercially and is any one of maize, wheat, rice, potato, cassava, peanut, beans, carrots, tomato or tobacco crop for example.

Preferably ADPG PPase activity is increased by the method of the invention.

An increase in starch content, especially in potatoes, may be measured as an increase in specific gravity (S.G.) of the plant or tuber, for example.

Preferably the plasmid incorporates a homologue of the *brittle-2* gene of an enzyme catalysing starch synthesis. Alternatively, the plasmid may incorporate a homologue of the *shrunken-2* gene of an enzyme catalysing starch synthesis.

In order that the present invention may be easily understood and readily carried into effect reference will now be made to the following Example and the drawings, in which:

Figure 1a shows a transformation vector or plasmid containing the *brittle-2* gene,

Figure 1b shows a transformation vector or plasmid containing the *shrunken-2* gene,

Figure 2a shows a Southern blot of DNA extracted from treated and untreated plants,

Figure 2b shows a Northern blot of RT-PCR products from treated and untreated plants,

Figure 3a is a graph of ADPG PPase activity against lines containing the *brittle-2* and *shrunken-2* genes,

Figure 3b is a graph of ADPG PPase activity against lines containing the *brittle-2* gene,

Figure 4 shows in graphical form the specific gravity of tubers as the cumulative freuquency of tubers in four

classes of ADPG PPase activity for lines transformed with *brittle-2* and *shrunken-2* genes, and

Figure 5 shows starch synthesis against four lines of different ADPG PPase activity.

Transgenic potato plants were produced containing a gene from wheat which is homologous to the *brittle-2* gene in maize. This gene is thus known as the wheat *brittle-2* gene. We found that surprisingly expression of the *brittle-2* gene in transgenic potato plants caused an increase in the ADPG PPase activity. It thus appears possible to increase the activity of this enzyme in the cell by expressing only one of the two subunit proteins required to make an active enzyme. When the activity of ADPG PPase is a major factor in limiting the amount of starch made or stored in a plant, then the expression of just *brittle-2* protein provides a mechanism of increasing the amount of starch in the tuber and the specific gravity in the tuber, and possibly increasing the amount of starch in any plant that stores starch. This would improve the yield of starch from the plant and would be of great commercial value.

The transgenic potato plants transformed with the gene for the *brittle-2* subunit of ADPG PPase from wheat were analysed to identify the presence of the subunit protein in the transgenic potato plants and the degree of enzyme (ADPG PPase) activity in the plants. The amount of starch in the plants can also be assessed. The standard methods used in these analyses are described below:

Production of Transgenic Potato Plants

For the purpose of the present invention a coding sequence is selected which when expressed in transgenic plants causes an increase in ADPG PPase activity. The coding sequence may be from any plant. For the purpose of example the wheat homologue of the *brittle-2* locus is chosen (Ainsworth, C; Tarvis,M; Clark,J. Pl. Mol. Biol. 23 23-33; 1993 Isolation and analysis of a cDNA encoding the small subunit of ADP-glucose pyrophosphorylase from wheat). This may be inserted into a transformation vector as shown in Figure 1a. This plasmid pfW4091 was deposited under the Budapest treaty for the International Recognition of the Deposit of Micro-organisms for the purposes of Patent Procedure, at the National Collection of Industrial and Marine Bacteria on 13 June 1994 under accession number NCIMB40649. The similar plasmid pfW 4151 containing the *shrunken-2* coding sequence Figure 1b was deposited on 13 June 1994 under accession number NCIMB40650. The vector may therefore comprise one or more operative genes, a selectable marker gene and these may be introduced between the T-DNA borders. The operative genes consist of a promoter sequence to cause expression of the gene in tubers or other starch storing organs, tissues or cells, the coding sequence and the terminator sequence.

The vector is therefore typically provided with transcriptional regulatory sequences and/or, if not present at the 3'-end of the coding sequence of the gene, a stop codon. A DNA fragment may therefore also incorporate a terminator sequence and other sequences

which are capable of enabling the gene to be expressed in plant cells. An enhancer or other element able to increase or decrease levels of expression obtained in particular parts of a plant or under certain conditions, may be provided in the DNA fragment and/or vector. The vector is also typically provided with an antibiotic resistance gene which confers resistance on transformed plant cells, allowing transformed cells, tissues and plants to be selected by growth on appropriate media containing the antibiotic.

Transformed plant cells can be selected by growth in an appropriate medium. Plant tissue can therefore be obtained comprising a plant cell which harbours a gene encoding an enzyme under the control of a promoter, for example in the plant cell genome. The gene is therefore expressible in the plant cell. Plants can then be regenerated which include the gene and the promoter in their cells, for example integrated in the plant cell genome such that the gene can be expressed. The regenerated plants can be reproduced and, for example, seed obtained.

A preferred way of transforming a plant cell is to use *Agrobacterium tumefaciens* containing a vector comprising a chimaeric gene as above. A hybrid plasmid vector may therefore be employed which comprises:

(a) a chimaeric gene containing regulatory elements capable of enabling the gene to be expressed when integrated in the genome of a plant cell;

(b) at least one DNA sequence which delineates the DNA to be integrated into the plant genome; and

(c) a DNA sequence which enables this DNA to be transferred to the plant genome.

Typically the DNA to be integrated into the plant cell genome is delineated by the T-/DNA border sequences of a Ti-plasmid. If only one border sequence is present, it is preferably the right border sequence. The DNA sequence which enables the DNA to be transferred to the plant cell genome is generally the virulence (vir) region of a Ti-plasmid.

The gene coding for the polypeptide and its transcriptional and translational control elements can therefore be provided between the T-DNA borders of a Ti-plasmid. The plasmid may be a disarmed Ti-plasmid from which the genes for tumorigenicity have been deleted. The gene and its transcriptional control elements can, however, be provided between T-DNA borders in a binary vector in trans with a Ti-plasmid with a vir region. Such a binary vector therefore comprises:

(a) the chimaeric gene under the control of regulatory elements capable of enabling the gene to be expressed when integrated in the genome of a plant cell; and

(b) at least one DNA sequence which delineates the DNA to be integrated into the plant genome.

Agrobacterium tumefaciens, therefore, containing a hybrid plasmid vector or a binary vector in trans with a Ti-plasmid possessing a vir region can be used to

transform plant cells. Tissue explants such as stems or leaf discs may be inoculated with the bacterium. Alternatively, the bacterium may be co-cultured with regenerating plant protoplasts. Plant protoplasts or tissues may also be transformed by direct introduction of DNA fragments which encode the enzyme and in which the appropriate transcriptional and translational control elements are present or by a vector incorporating such a fragment. Direct introduction may be achieved using electroporation, polyethylene glycol, microinjection or particle bombardment.

Plant cells from angiospermous, gymnospermous, monocotyledonous or dicotyledonous plants can be transformed according to the present invention. Monocotyledonous species include barley, wheat, maize and rice. Dicotyledonous species include cotton, cassava, lettuce, melon, pea, petunia, potato, rape, soyabean, sugar beet, sunflower, tobacco and tomato. Potato cultivars to which the invention is applicable include Desiree, Maris Bard, Record, Russet Burbank, Atlantic and Pentland Dell.

Tissue cultures of transformed plant cells are propagated to regenerate differentiated transformed whole plants. The transformed plant cells may be cultured on a suitable medium, preferably a selectable growth medium. Plants may be regenerated from the resulting callus. Transgenic plants are thereby obtained whose cells incorporate the chimaeric gene in the genome, the chimaeric gene being expressible in the cells of the

plants. Seed or other propagules from the regenerated plants can be collected for future use.

A preferred procedure in respect of the potato variety Record and Desiree is as follows.

Plant Material

Potato shoot cultures are maintained *in vitro* on Murashige and Skoog (MS) medium in Magenta GA-7 containers at 22°C (16h/8h light/dark). These are nodally sub-cultured every 3 weeks.

In vitro shoots of 2-3 inches (5-7.5cm) height are potted in 2.5 inches (6.4cm) pots of Levingtons F1 compost. They are weaned in a propagator for one week in a growth room at 18°C (16h/8h light/dark). The propagator is removed and the plants repotted at 3 weeks into 5 inch (12.7cm) pots. At 5-7 weeks the plants are used for tranformation.

Agrobacterium Tumefaciens

Liquid overnight cultures of suitable strains, e.g. LBA4404, C58#3 are grown at 28°C to an OD_{600} of 0.8 in L-broth (see appendix).

Cocultivation

The youngest four most expanded leaves are taken and surface sterilised in 10% Domestos (commercial bleach) for 15 minutes. Leaves are rinsed thoroughly with sterile water and then cut into discs with a 7mm cork borer. The discs are mixed with the Agrobacterium for 1-5 minutes, blotted dry on filter paper (Whatman No. 1) and then placed on callusing medium (see appendix) in 90mm triple vented petri dishes, lower epidermis down. The 90mm

triple vented petri dishes are sealed with tape, cut to allow gas exchange and then incubated at 22°C/(16h/8h light/dark). The discs are transferred to callusing medium plus 500μg ml^{-1} of claforan and 30μg ml^{-1} kanamycin after 48 hours. This removes bacteria and selects for transformed cells.

<u>Regeneration of Transformed Shoots</u>

After 1 week, the discs are transferred to shooting medium (see appendix) containing the same antibiotics. Further transfers are made onto the same medium until shoots can be excised (usually about 4 weeks). Shoots with calli are transferred to MS medium with cefotaxime (500μg/ml) in well ventilated containers, e.g. Magenta. Transformants are maintained, after several passages with cefotaxime to remove bacteria, on MS medium. They may be removed from *in vitro*, weaned and grown to maturity as described for the stock plants. The process yields transformed potato plants at a frequency of up to 30% of the discs cocultivated.

Appendix

L-broth	10g l^{-1} bactotryptone 5g l^{-1} yeast extract 5g l^{-1} sodium chloride 1g l^{-1} glucose
Callusing medium	MS with 3% sucrose 0.5mg l^{-1} 2,4-D 2.5mg l^{-1} BAP
Shooting medium	MS plus 3% sucrose 2.5mg l^{-1}BAP 1.0mg l^{-1}GA_3

<u>Identification of wheat *Brittle-2* gene and expression in Transgenic Plants</u>.

A southern blot was prepared with potato DNA extracted from lines transformed with NCIMB 40649 and lines transformed with NCIMB 40649 and NCIMB 40650 together. The extracted plant DNA was restricted with *Hind*III and 10µg of DNA was used per track of the 1% agarose gel. The blot was probed with the *brittle-2* coding sequence obtained from *Bam*HI restricted plasmid DNA of NCIMB 40649. The blot was hybridised overnight at 55°C in 5xSSC. After washing to a stringency of 0.2xSSC at 55°C the blot was autoradiographed. The result shown in Figure 2a indicates that between one and four copies of the *brittle-2* gene had been introduced into the plants.

In Figure 2a lanes 1-4 are of DNA extracted from plants transformed with both the *brittle-2* and *shrunken-2* genes. Lanes 5-13 show DNA extracted from lines independently transformed with just the *brittle-2* gene. Lane 14 shows DNA from an untransformed potato plant.

To show that DNA was expressed as message RNA oligonucleotide primers were prepared for the procedure known as RT-PCR which was performed on mRNA extracted from tubers of transformed potato plants. RT-PCR was performed on mRNA extracted from tuber material by the method described by Shirzadegan *et al*. (Nucleic acid research <u>19</u> 6055; 1991 An efficient method of isolation of RNA from tissue cultured plant cells). The mRNA was treated with DNAse to remove contaminating DNA. For first strand synthesis the primer ATA ATC ATC GCA AGA CCG GCA ACA GGA

was used at 42°C for 100 minutes. After removal of RNA with RNAse the second strand was synthesised to obtain a fragment at the 5' end and a fragment at the 3' end of the *brittle-2* cDNA. To amplify the 5' end the primers CCT CGT CAG GGG ATA CAA TCT AGT CCC and CAC CAA CAA AAT TTC GCG GAT CC were used and to amplify the 3' end the primers CAG ACC ATG CTA TTT GTT G and ATA ATC ATC GCA AGA CCG GCA ACA GGA were used. The conditions of amplification were of 24 cycles of 1 minute at 94°C, 30 seconds at 50°C, and 3 minutes at 72°C. After separation of the products on a 1% agarose gel and Southern blotting the blot was probed as described above. The results in Figure 2b show that the introduced gene was expressed as mRNA.

In Figure 2b lanes 1-4, 5-8, 9-12 show RT-PCR products from three lines transformed with the *brittle-2* sequence. Even numbered lanes show reactions lacking reverse transcriptase to indicate DNA contamination of the RNA. Lanes 1,2,5,6,9 and 10 show amplification of the 3' end and lanes 3,4,7,8,11,12 show amplification of the 5' end. When no RNA or a non-transgenic plant was used as a control, no signal was obtained.

PRODUCTION OF ANTISERA TO IDENTIFY ADPG PPASE IN PLANTS

1. Preparation of proteins from *E. coli* expression vectors.

E. coli cells, transformed with GEX2T (Phamacia Ltd) expression constructs were grown up in the following way: A 1l-flask containing 100ml of LB broth (10g/l tryptone; 5g/l yeast extract; 10g/l sodium chloride (NaCl)) with 100 μg/ml ampicillin added, was inoculated with 20μl of *E.*

coli cells, and grown overnight at 37°C. The overnight culture was transferrred into a 5l-flask containing 900ml of LB broth and grown on for 1 hour. The cells were induced to express the fusion protein by adding isopropyl Beta-D-thiogalactopyranoside (IPTG) to a final concentration of 1mM. After growing for a further 4hours the cells were harvested by centrifuging at 7000rpm for 10 minutes. Pelleted cells were stored at -80°C prior to extraction.

The pelleted cells were resuspended in 90ml of ice cold 50mM N-tris(hydroxmethyl)aminoethane (Tris), 150mM NaCl, pH 8.0, placed in a glass beaker and sonicated for 45 seconds. Triton X-100 was added to a final concentration of 1%, and the extract clarified by centrifuging for 20 minutes at 10,000rpm and 4°C. After centrifuging, the supernatant was decanted into a 250ml-plastic bottle and 2-3ml of a 50% slurry of glutathione-sepharose affinity resin (Pharmacia Ltd), pre-equilibrated with 50mM Tris, 150mM NaCl, pH 8.0, was added and the bottle was gently rocked for 1-2 hours at room temperature. The resin was then loaded into a 5ml-column and washed sequentially with 50mM Tris, 150mM NaCl, 1% Triton X-100, pH 8.0 and then 50 mM Tris, 150mM NaCl, pH 8.0, until no further protein was detected in the washings. The bound fusion protein was then eluted from the resin with 50mM Tris, 150mM NaCl, 5mM reduced glutathione, pH 8.0. Fractions (1ml) were collected and analysed for protein content using the Biorad dye-binding assay for protein (Bradford, (1976) Analytical

Biochemistry, 72, pg. 248-254). Fractions showing the peak of protein were bulked prior to further analysis.

2. Antiserum production

A fusion protein, consisting of the wheat *brittle-2* protein sequence linked to glutathione-s-transferase was prepared from transformed *E. coli* cells as described above. This preparation of protein was dialysed against three changes of 50mM Tris, pH 8.0 and made up to three aliquots, one of 100 μg of protein and two of 50μg of protein, in 500μl of 50mM Tris, pH 8.0. The 100μg aliquot was mixed with an equal volume of Freund's complete adjuvant and injected subcutaneously into the flank of a New Zealand white rabbit. Each of the two 50μg aliquots of protein were mixed with an equal volume of Freund's incomplete adjuvant and injected into the same rabbit 4 and 8 weeks after the initial injection. Blood was collected 12 weeks after the primary injection and the cells separated from the serum by clotting and centrifugation. The serum was retained and stored at -20°C.

IDENTIFICATION OF WHEAT *BRITTLE-2* PROTEIN IN TRANSGENIC PLANTS

The following procedure was used to identify wheat *brittle-2* protein accumulated in tubers of transformed potato plants.

1. Sodium dodecylsulphate-polyacrylamide gel electrophoresis.

Electrophoresis of protein samples was routinely performed using the Schagger and von Jagow system

(Analytical Biochemistry (1987), 166, pg. 368-379). Protein extracts were prepared by homogenising tuber tissue (50-100mg) in an extraction buffer consisting of 50mM N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (Hepes), pH 8.0; 10mM diaminoethane tetra-acetic acid (EDTA); 10mM dithiothreitol (DTT). Protein samples, containing up to 100µg of protein were prepared by precipitating with acetone, followed by resuspension in water (50µl) and 2X sample loading buffer (50µl). Samples were boiled for 60 seconds prior to loading on the gel and were subjected to electrophoresis at 50-60V (constant) for approximately 20 hours. 2X sample loading buffer consisted of 100mM Tris, pH 6.8; 8% (w/v) sodium dodecylsulphate (SDS); 24% (w/v) glycerol; 4% (v/v) beta-mercaptoethanol; 0.02% (w/v) Coomassie blue.

2. Electroblotting of proteins

Proteins separated by SDS polyacrylamide gel electrophoresis were transferred onto Immobilon-P PVDF membrane (Millipore) by electroblotting. Membrane, Whatman 3mm paper and sponges were pre-equilibrated in transfer buffer (25mM Tris; 192mM glycine; 20% methanol; pH 8.3) before use. Gels were placed in close contact with membrane, and assembled into transfer cassettes in the specific arrangement given in the manufacturers instructions. Cassettes were placed into an electroblotting tank containing transfer buffer and transfer of proteins from gel to membrane facilitated by applying 50V at 4°C for 3-4 hours. Blotting was monitored

by using prestained protein molecular weight markers (Sigma Chemical Co.).

3. Immunodetection of immobilised proteins

Specific proteins were detected on Immobilon-P membranes by using antibodies raised against proteins expressed in *E. Coli*. Membranes were taken directly from the electroblotting tank and placed in a glass dish. The membranes were rinsed briefly with phosphate buffered saline (PBS, 10mM sodium dihydrogen phosphate (NaH_2PO_4); 150mM NaCl; pH 7.2) and the remaining protein binding sites were blocked by treating with 4% (w/v) bovine serum albumin (BSA) in PBS for 30 minutes. Then membranes were challenged with primary antibody, at a suitable dilution (typically 1/1000-1/10000 (v/v)) in PBS containing 4% BSA for 16 hours at room temperature and with gentle shaking. Excess primary antibody was removed by washing the membranes with several changes of PBS. Membranes were then treated with 20-40μl of alkaline phosphatase conjugated anti-rabbit IgG (immunoglobulin G) in up to 200ml of PBS containing 2% (w/v) BSA for 2-3 hours. Unbound conjugate was removed after incubation by washing with several changes of 1% (v/v) Triton X-100 in PBS. Membranes were then washed briefly with 100mM diethanolamine buffer, pH 9.8 and developed by incubating with alkaline phosphatase reaction mixture (120μM nitroblue tetrazolium; 135μM 5-Bromo-4-chloroindolyl phosphate; 4mM magnesium chloride ($MgCl_2$); 100mM diethanolamine; pH 9.8). Reaction was allowed to occur until purple-blue bands were visualised, usually after 15-

30 minutes. Reaction was stopped by rinsing the membranes under reverse osmosis (RO) water. Membranes were allowed to dry face down on filter paper and stored in the dark.

ASSAY OF ADPG PPASE IN TRANSGENIC POTATOES

1. Preparation of extracts

Potato tuber tissue, 2-3g, was homogenised with 3ml of extraction buffer (50mM Hepes, pH 8.0; 10mM EDTA; 10mM DTT; 10% (w/v) BSA) using a pestle and mortar. The extract was clarified by centrifugation. To de-salt the extract 2.5ml of the clarified extract was loaded onto a PD10 gel filtration column (Pharmacia Ltd) pre-equilibrated with extraction buffer, and eluted with 3.5ml of extraction buffer. This preparation was taken for enzyme assay.

2. Enzyme Assay

The principle of the enzyme assay is as follows:

$$\text{ADPG + PPi} \xrightarrow[\text{ADPG PPase}]{} \text{glucose 1-phosphate + ATP}$$

$$\text{glucose 1-phosphate} \xrightarrow{\text{Phosphoglucomutase}} \text{glucose 6-phosphate}$$

$$\text{glucose 6-phosphate} \xrightarrow[\text{NAD} \rightarrow \text{NADH}]{\text{Glucose-6-phosphate dehydrogenase}} \text{6-phosphogluconate} + CO_2$$

NADH was detected spectrophotometrically at 25°C and 340nm.

To a plastic cuvette, in a final volume of 1ml was taken:

40mM Hepes, pH 8.0
10mM magnesium chloride ($MgCl_2$)
1mM tetra-sodium pyrophosphate ($Na_4P_2O_7$)
0.4mM nicotinamide adenine dinucleotide (NAD)
4 units glucose-6-phosphate dehydrogenase
2 units phosphoglucomutase
24μM glucose 1,6-diphosphate (Glc-1,6-P_2)
up to 300μl of extract

The reaction was started by adding adenosine diphosphoglucose (ADPG) to a final concentration of 0.8mM.

ANALYSIS OF SPECIFIC GRAVITY OF TRANSGENIC POTATOES

Whole tubers were weighed in air and under water. The specific gravity was calculated as:

$$\frac{\text{weight in air}}{\text{weight in air - weight in water}}$$

ANALYSIS OF STARCH CONTENT OF TRANSGENIC POTATOES

Tuber tissue (40-70mg) was extracted in 500μl of 45% $HClO_4$. An aliquot of this extract (50μl) was made up to 1ml with 400mM Hepes, pH 8.0 and then split into two 500μl portions which were both made up to 1ml by the addition of 400mM Hepes, pH 8.0. To one portion was then added 100 units alpha-amylase and 7 units amyloglucosidase, no enzymes were added to the other portion and both were left overnight before assaying for glucose.

The glucose assay was performed spectrophotometrically at 25°C and 340nm.

To a plastic cuvette, in a final volume of 1ml was taken:

100mM Hepes, pH 8.0
4mM $MgCl_2$
4mM NAD
3mM adenosine triphosphate (ATP)
3 units glucose-6-phosphate dehydrogenase from Leuconostoc (Boehringer Mannheim)
500-300 microlitres of starch digest.

The reaction was started with 0.3 units of yeast hexokinase (Boehringer Mannheim).

The amount of starch present in the potato tissue can be calculated from the amount of glucose measured in the assay.

The following results were obtained using the above methods.

1. Recognition of proteins by anti-*Brittle-2* antiserum in extracts of potato and wheat

Extracts of potato tuber and wheat endosperm tissue were prepared according to the methods. Aliquots, containing 100μg of protein were taken and run on SDS-PAGE gels as described, blotted and challenged with the anti-*brittle-2* antiserum. At a dilution of 1/10000, only one protein band was detected in tracks corresponding to the wheat and potato extracts. Furthermore, the potato band was distinguishable from the wheat band because they were of different sizes. In a third extract, made up of both wheat and potato tissue, two bands were distinguishable, corresponding to the sizes of the bands seen in the individual wheat and potato extracts.

2. Detection of proteins in tubers of potato plants transformed with the gene sequence for the wheat *brittle-2* gene.

Potato plants were transformed by the leaf disc co-cultivation method with *Agrobacterium tumefaciens*

containing the plasmid pFW 4091 containing the DNA coding for the wheat gene for the *brittle-2* protein of wheat. Further plants were transformed using the same method and a combination of *Agrobacterium tumefaciens* containing the plasmid pFW 4151 containing the DNA coding for the wheat gene for the *shrunken-2* protein of wheat and *Agrobacterium tumefaciens* containing the plasmid pFW 4091 containing the DNA coding for the wheat gene for the *brittle-2* protein of wheat. Plasmid pFW 4091 was deposited under accession number NCIMB 40649 and plasmid pFW 4151 was deposited under accession number NCIMB 40650 as described above. Potato tubers from plants which had been transformed with the DNA coding for the wheat gene for the *brittle-2* protein of wheat, were analysed for the expression of the gene by Western blotting, as described in the methods, using the antibody raised against the *brittle-2*/glutathione-s-transferase fusion protein. Similarly tubers from lines which had been transformed with *brittle-2* and *shrunken-2* from wheat were analysed. As described in section 1. above this antiserum recognises a single protein in wheat and a single protein in potato which are distinguishable from each other on the basis of their size. In this way lines were selected which were only expressing the wheat *brittle-2* protein. Tubers of these lines were assayed for ADPG PPase activity and starch content as described in the methods, and compared with the activities and starch contents of control tubers.

Fifty lines from tubers treated according to the inventive method were analysed and compared against fifty

lines from control (non-treated) tubers. Figures 3a and 3b show a selection of the extreme ranges of ADPG PPase activity (namomoles per minute per gram fresh weight) seen in lines which contained the chimaeric gene for *brittle-2* and *shrunken-2* (Figure 3a) and in lines containing the chimaeric gene for *brittle-2* (Figure 3b). We believe that these lines show significantly greater ADPG PPase activity than control tubers.

Figure 4 shows in graphical form the specific gravity of tubers as the cumulative frequency of tubers in four classes of ADPG PPase activity for lines transformed with *brittle-2* and *shrunken-2*. A similar analysis for lines transformed with just *brittle-2* gives the following change in the median value of the population:

		median specific gravity
transgenic with *brittle-2*	line 153	1.09
transgenic with *brittle-2*	line 32	1.095
Control	line 16	1.087
Control	line 28	1.087
Control	line 38	1.089

We believe that this increased ADPG production will also lead to increased starch content in the plants as measured by the above described method when grown under appropriate conditions or when suitable other genes are introduced (see below).

<u>Measurement of the Synthesis and Turnover to Starch</u>

To determine the effect of the change in activity on starch synthesis radiolabelled sucrose was supplied to developing tubers of transgenic plants with increased activity of ADPG PPase. Starch was extracted as described

above and the radioactivity determined by liquid scintillation counting. A line transformed with *brittle-2* gene with elevated ADPGppase activity was compared to a control line and gave the following result:

	% Total Counts incorporated into starch	
	mean	**sem**
brittle-2 line	0.52	0.37
control ine	0.30	0.18

sem = standard error of mean

To confirm this observation a further experiment with four lines showing different activities of ADPG PPase was used (see Figure 5). The results in Figure 5 show that the starch is more rapidly synthesised but show that under certain conditions the starch is more rapidly broken down. Therefore we suggest that this shows that for the invention to be universally applicable it is necessary to introduce operative genes to increase the activity of ADPG PPase and operative genes to decrease the activity of amylase (EC 3.2.1.1 and EC 3.2.1.2) and starch phosphorylase (EC 2.4.1.1).

CLAIMS

1. A method of increasing the enzyme activity in a plant comprising introducing into a plant one of the genes of one of the subunit proteins of an enzyme catalysing starch synthesis, thereby causing expression of the subunit gene in the plant to produce the subunit protein, and an increase in the enzyme activity in the plant cells.
2. A method according to Claim 1, and further comprising introducing one of the genes of one of the subunit proteins of a plurality of other enzymes catalysing starch synthesis.
3. A method according to Claim 1 or 2, wherein the gene is the *brittle-2* gene or a homologue thereof.
4. A method according to Claim 3, wherein the gene is the wheat brittle-2 gene.
5. A method according to Claims 1, 2, 3 or 4, wherein ADGP PPase activity is increased.
6. A method according to Claims 1, 2, 3, 4 or 5, wherein genes operable to decrease the activity of enzymes which degrade starch are also introduced into the plant.
7. A method according to Claim 6, wherein the genes operable to decrease the activity of enzymes degrading starch decrease the activity of amylase (E.C. 3.2.1.1 and E.C. 3.2.1.2) or starch phosphorylase (E.C. 2.4.1.1).
8. A method according to any one of the preceding claims, wherein the plant is a monocot selected from the group comprising wheat, barley, rye, maize or rice.

9. A method according to any one of Claims 1 to 7, wherein the plant is a dicot selected from the group comprising potato, tomato, cassava, peanut, bean or pea.

10. A method according to any one of the preceding claims, wherein an increase in starch content is measured by an increase in specific gravity of the plant.

11. A plant into which has been introduced one of the genes of one of the subunit proteins of an enzyme catalysing starch synthesis, which plant expresses the gene to produce a subunit protein, and increases the enzyme activity in the plant cells.

12. A plant according to Claim 11 and further comprising one of the genes of one of the subunit proteins of a plurality of other enzymes catalysing starch synthesis.

13. A plant according to Claim 11 or 12 and being a monocot selected from the group comprising wheat, barley, rye, maize or rice.

14. A plant according to Claim 11 or 12 and being a dicot selected from the group comprising potato, tomato, cassava, peanut, bean or pea.

15. A plasmid incorporating one of the genes of one of the subunit proteins of an enzyme catalysing starch synthesis in plants.

16. A plasmid according to Claim 15, which plasmid also contains one of the genes of one of the subunit proteins of one or more other enzymes catalysing starch synthesis in the plants.

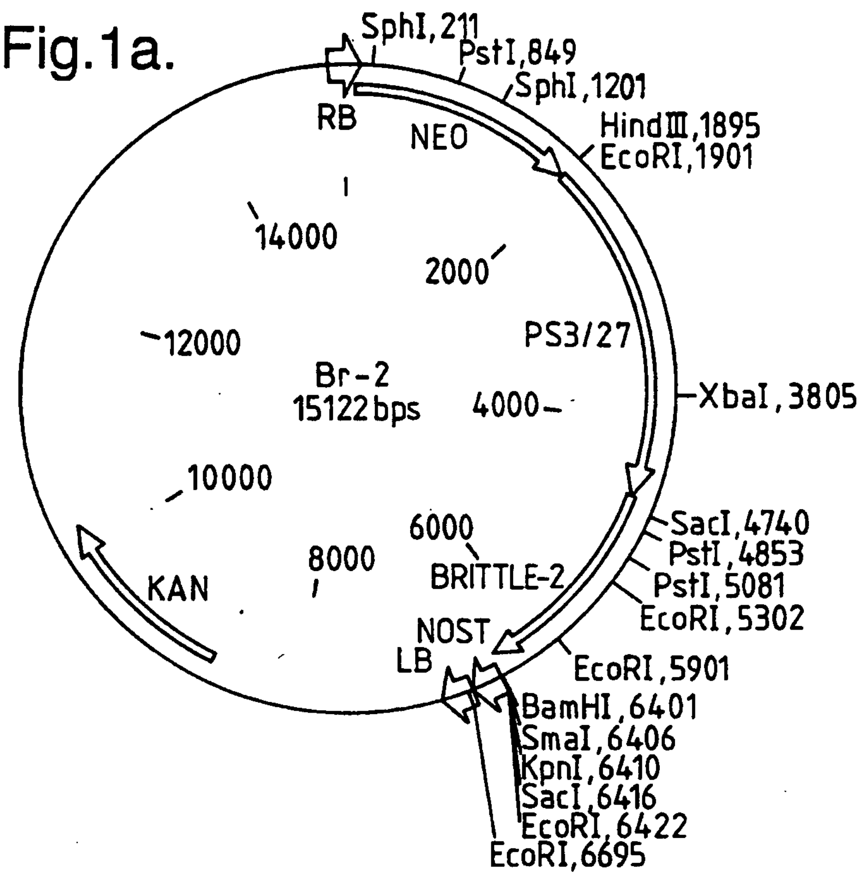
17. A plasmid according to Claim 15 or 16, wherein the gene is a *brittle-2* gene or homologue thereof.

18. A plant cell harbouring a plasmid according to Claim 15 or 16.

19. A method of increasing the enzyme activity in a plant substantially as described herein with reference to Figures 2a, 2b, 3a, 3b and 4 hereof.

20. A plasmid incorporating a gene substantially as described herein with reference to Figures 1 and 2 hereof.

Fig.1a.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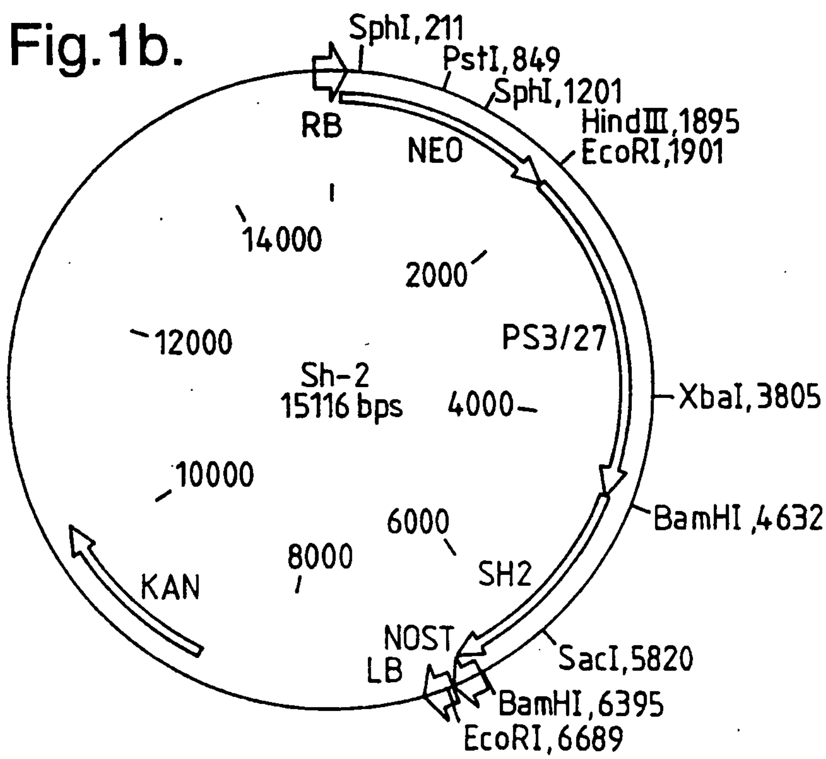
HindIII,1895
EcoRI,1901
RB
NEO
14000
2000
12000
PS3/27
Br-2
15122 bps
4000
XbaI,3805
10000
6000
SacI,4740
PstI,4853
PstI,5081
EcoRI,5302
8000
BRITTLE-2
KAN
NOST
LB
EcoRI,5901
BamHI,6401
SmaI,6406
KpnI,6410
SacI,6416
EcoRI,6422
EcoRI,6695

Fig.1b.


SphI,211
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EcoRI,1901
RB
NEO
14000
2000
12000
PS3/27
Sh-2
15116 bps
4000
XbaI,3805
10000
6000
BamHI,4632
8000
SH2
KAN
NOST
LB
SacI,5820
BamHI,6395
EcoRI,6689

Fig.2a.

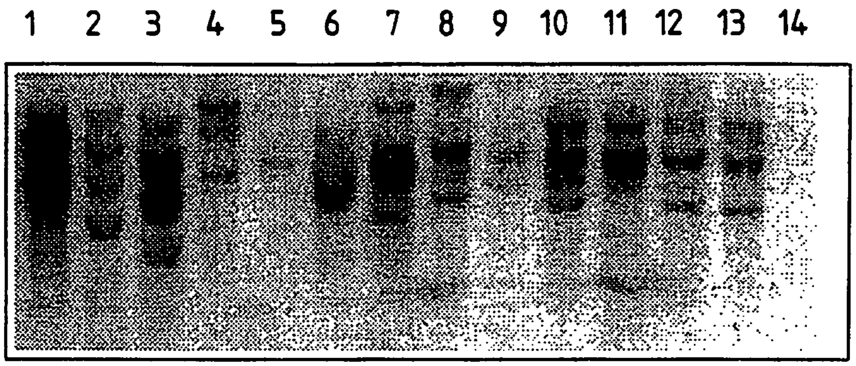


Fig.2b.

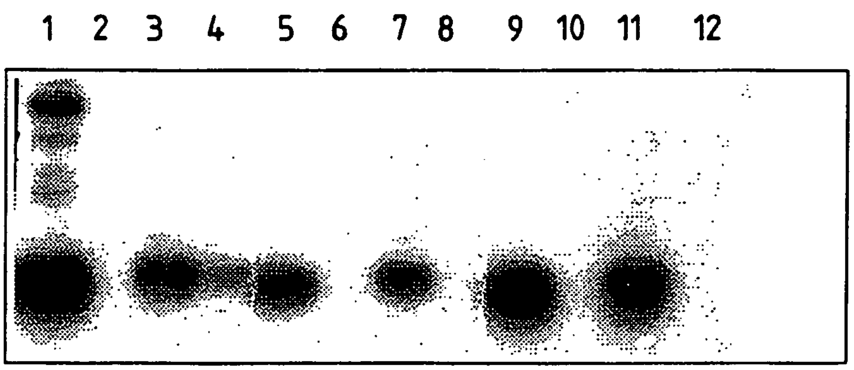


Fig.3a.

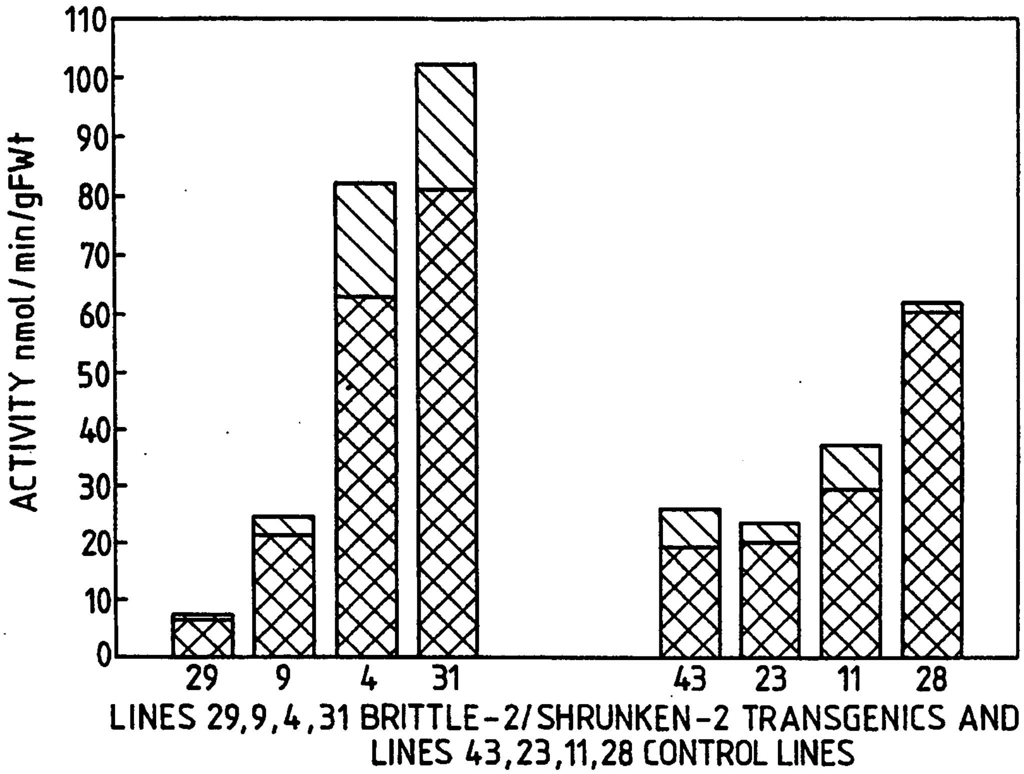

110
100
90
80
70
60
50
40
30
20
10
0
ACTIVITY nmol/min/gFW†
29
9
4
31
43
23
11
28
LINES 29,9,4,31 BRITTLE-2/SHRUNKEN-2 TRANSGENICS AND
LINES 43,23,11,28 CONTROL LINES

Fig.3b.

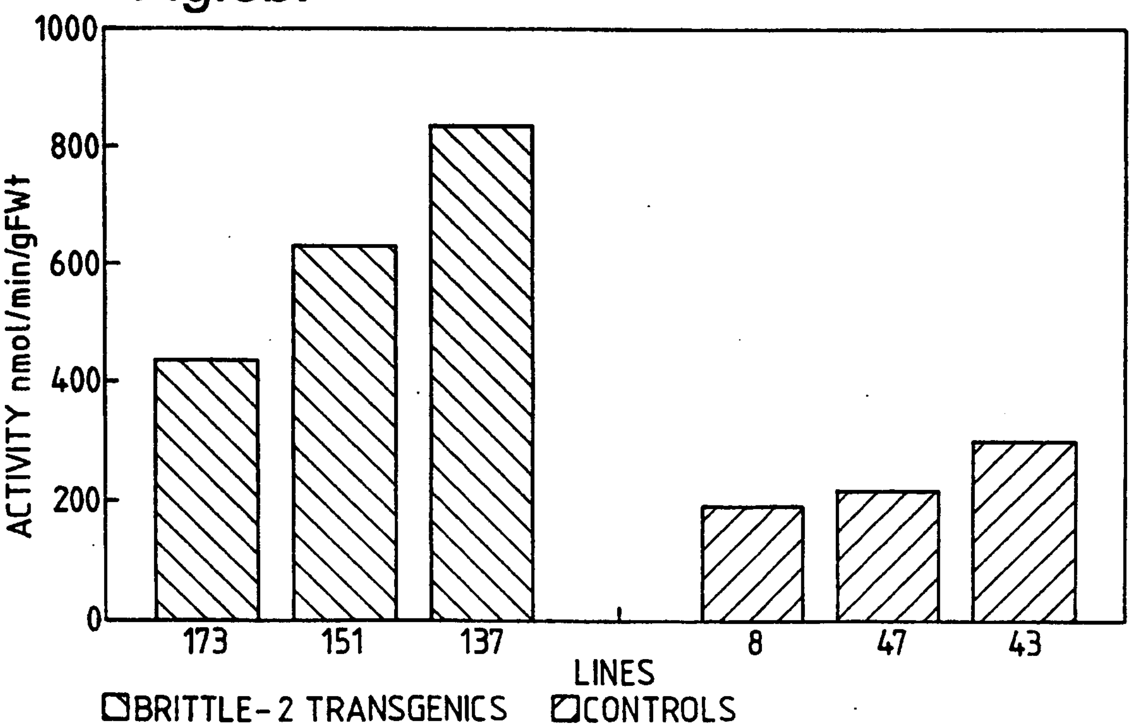

1000
800
600
400
200
0
ACTIVITY nmol/min/gFW†
173
151
137
8
47
43
LINES
BRITTLE-2 TRANSGENICS
CONTROLS

Fig.4.

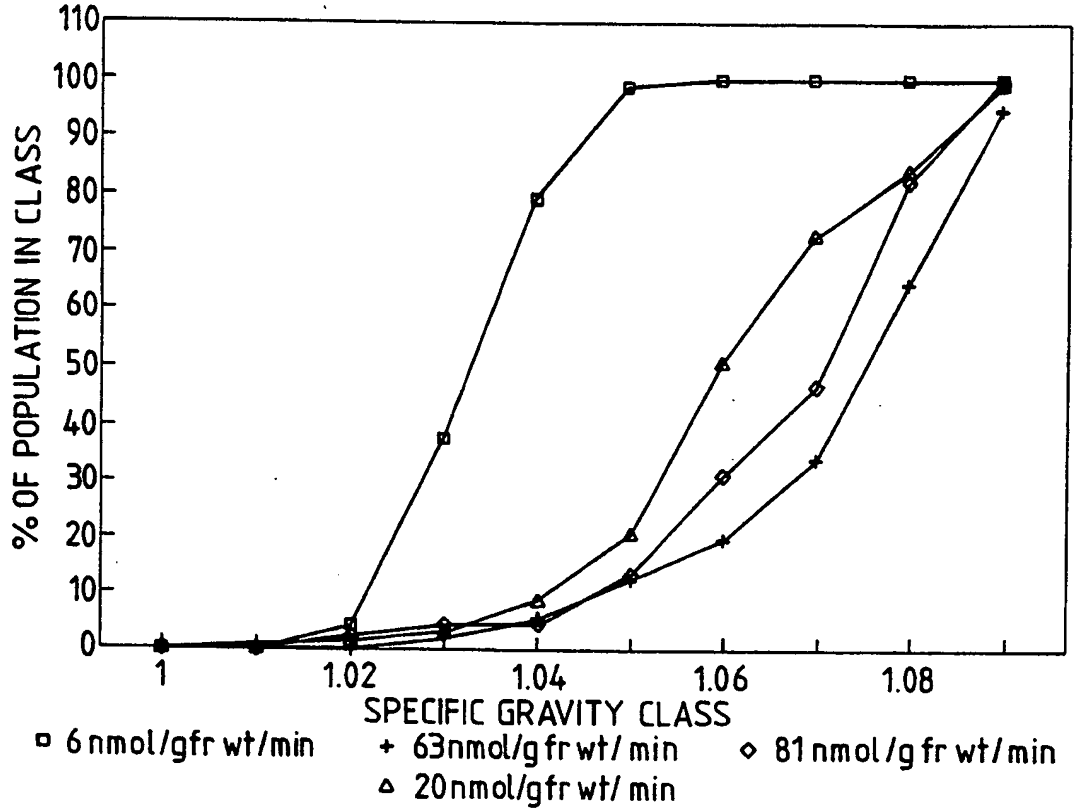
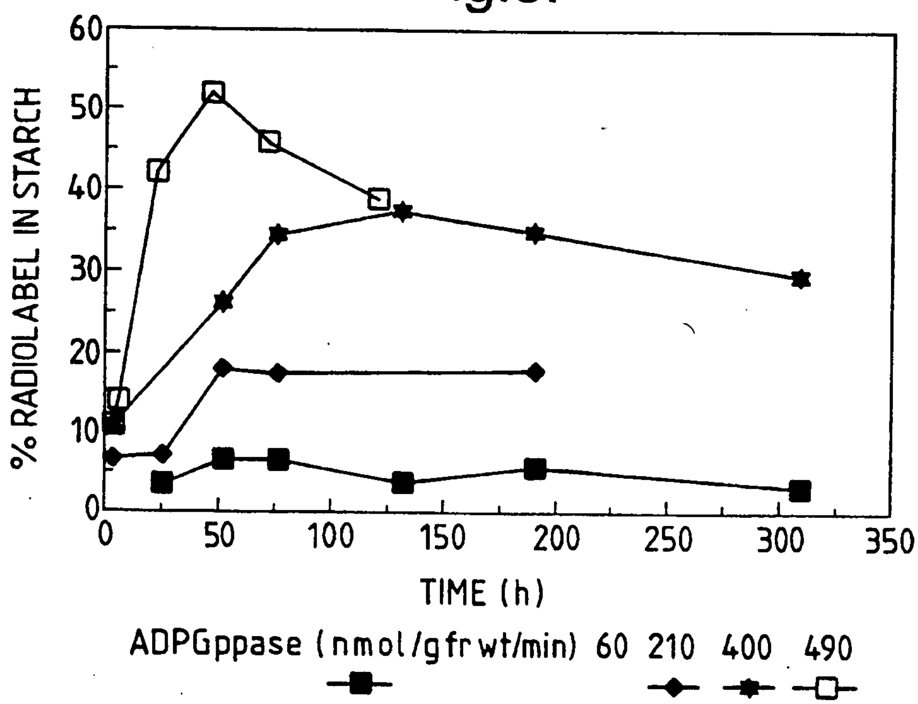

% OF POPULATION IN CLASS
110
100
90
80
70
60
50
40
30
20
10
0
1
1.02
1.04
1.06
1.08
SPECIFIC GRAVITY CLASS
6 nmol/gfr wt/min
63nmol/g fr wt/ min
81 nmol/g fr wt/min
20nmol/gfr wt/ min

Fig.5.


% RADIOLABEL IN STARCH
60
50
40
30
20
10
0
0
50
100
150
200
250
300
350
TIME (h)
ADPGppase (nmol/gfr wt/min) 60 210 400 490

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 95/01307

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/54 C12N15/82 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO-A-93 09237 (SANDOZ AG ;SANDOZ AG (DE); SANDOZ LTD (CH)) 13 May 1993 see examples 2,8	1,3,5,8, 11,13, 15,17-20
X	WO-A-94 11520 (ZENECA LTD ;KEELING PETER LEWIS (GB)) 26 May 1994 see page 15, line 4 - line 20; example 2D	1,3,5,8, 9,11-15, 18-20
X	WO-A-94 09144 (ZENECA LTD) 28 April 1994 see page 43, line 10 - page 45, line 15 see page 50, line 20 - page 53	1,8,9, 11-15,18

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
12 September 1995	06. 10. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Maddox, A

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PLANT PHYSIOLOGY, vol. 104, April 1994 pages 1159-166, SHEWMAKER, C.K., ET AL. 'EXPRESSION OF ESCHERICHIA COLI GLYCOGEN SYNTHASE IN THE TUBERS OF TRANSGENIC POTATOES (SOLANUM TUBEROSUM) RESULTS IN A HIGHLY BRANCEHD STARCH' see the whole document ---	1,9,11, 14,15,18
X	WO-A-91 19806 (MONSANTO CO) 26 December 1991 see the whole document ---	1,5, 9-11,14, 15,18
X	EP-A-0 368 506 (ICI PLC) 16 May 1990 see claim 13 ---	1,5,11, 15,18
X	WO-A-92 11382 (CALGENE INC) 9 July 1992 see the whole document ---	1,9,11, 15,18
X	BRITISH CROP PROTECTION COUNCIL MONOGRAPH, NO. 55, 1993 pages 197-201, BURRELL, M.M., ET AL. 'PROSPECTS FOR THE MODIFICATION OF METABOLISM' see page 199 ---	1,9,11, 14,15,18
X	IAPTC CONGRESS AMSTERDAM JUNE 24-29, 1990, page 177 VAN DER LEIJ, F.R., ET AL. 'EXPRESSION OF THE GENE ENCODING GRANULE BOUND STARCH SYNTHASE AFTER INTRODUCTION IN AN AMYLOSE-FREE AND A WILDTYPE POTATO (SOLANUM TUBEROSUM)' see abstract A5-28 ---	1,11,15, 16,18
X	THEORETICAL AND APPLIED GENETICS, vol. 88, June 1994 pages 369-375, FLIPSE, E., ET AL. 'EXPRESSION OF A WILD-TYPE GBSS GENE INTRODUCED INTO AN AMYLASE-FREE POTATO MUTANT BY AGROBACTERIUM TUMEFACIENS AND THE INHERITANCE OF THE INSERTS AT THE MICROSCOPIC LEVEL' see the whole document ---	1,11,16, 18

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1

Inte onal Application No
PCT/GB 95/01307

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PLANT MOLECULAR BIOLOGY, vol. 23, 1993 pages 23-33, AINSWORTH, C., ET AL. 'ISOLATION AND ANALYSIS OF A CDNA CLONE ENCODING THE SMALL SUBUNIT OF ADP-GLUCOSE PYROPHOSPHORYLASE FROM WHEAT' see the whole document	15,18
P,X	WO-A-95 09242 (SAGISAKA SHONOSUKE) 6 April 1995 see the whole document	1,5
P,X	WO-A-94 24292 (DANISCO) 27 October 1994 see the whole document	1,3,5,9, 11,14, 15,17,18
P,X	WO-A-94 28149 (MONSANTO CO ;BARRY GERARD FRANCIS (US); KISHORE GANESH MURTHY (US)) 8 December 1994 see the whole document	1,5,9, 11,14, 15,18
P,X	WO-A-94 28146 (HOECHST SCHERING AGREVO GMBH ;HESSE HOLGER (DE); MUELLER ROEBER BE) 8 December 1994 see claims 6,7; example 1	1,3,5,9, 11,15, 17,18

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 95/01307

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9309237	13-05-93	AU-A-	2920392	07-06-93
		EP-A-	0611395	24-08-94
		HU-A-	66207	28-10-94
		JP-T-	7500965	02-02-95
WO-A-9411520	26-05-94	AU-B-	5428594	08-06-94
WO-A-9409144	28-04-94	AU-B-	2696492	09-05-94
		EP-A-	0664835	02-08-95
WO-A-9119806	26-12-91	AU-B-	644203	02-12-93
		AU-A-	8220291	07-01-92
		EP-A-	0536293	14-04-93
EP-A-0368506	16-05-90	AU-B-	635904	08-04-93
		AU-A-	4430789	16-08-90
		EP-A-	0654531	24-05-95
		JP-A-	2273177	07-11-90
WO-A-9211382	09-07-92	US-A-	5349123	20-09-94
		EP-A-	0542929	26-05-93
WO-A-9509242	06-04-95	JP-A-	7099987	18-04-95
WO-A-9424292	27-10-94	AU-B-	6539294	08-11-94
WO-A-9428149	08-12-94	AU-B-	6947394	20-12-94
WO-A-9428146	08-12-94	DE-A-	4317596	01-12-94